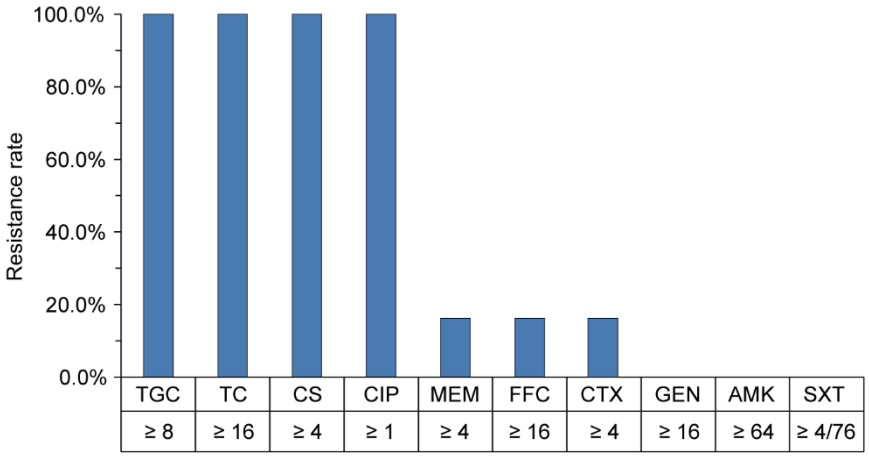

Figure S1. Resistance rates of ten antibiotics against 37 *tet*(X)-positive *Empedobacter* sp. strains. The resistance breakpoints of different antibiotics are also present. TGC, tigecycline; TC, tetracycline; CS, colistin; CIP, ciprofloxacin; MEM, meropenem; FFC, florfenicol; CTX, cefotaxime; GEN, gentamicin; AMK, amikacin; SXT, trimethoprim/sulfamethoxazole.

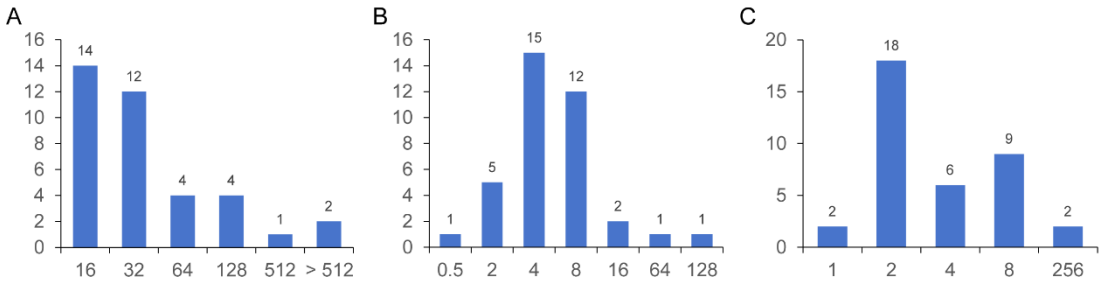

Figure S2. MICs of tylosin (A), tilmicosin (B), and tildipirosin (C) against 37 *tet*(X)-positive *Empedobacter* sp. strains. Bacterial numbers per dilution (mg/L) are present.

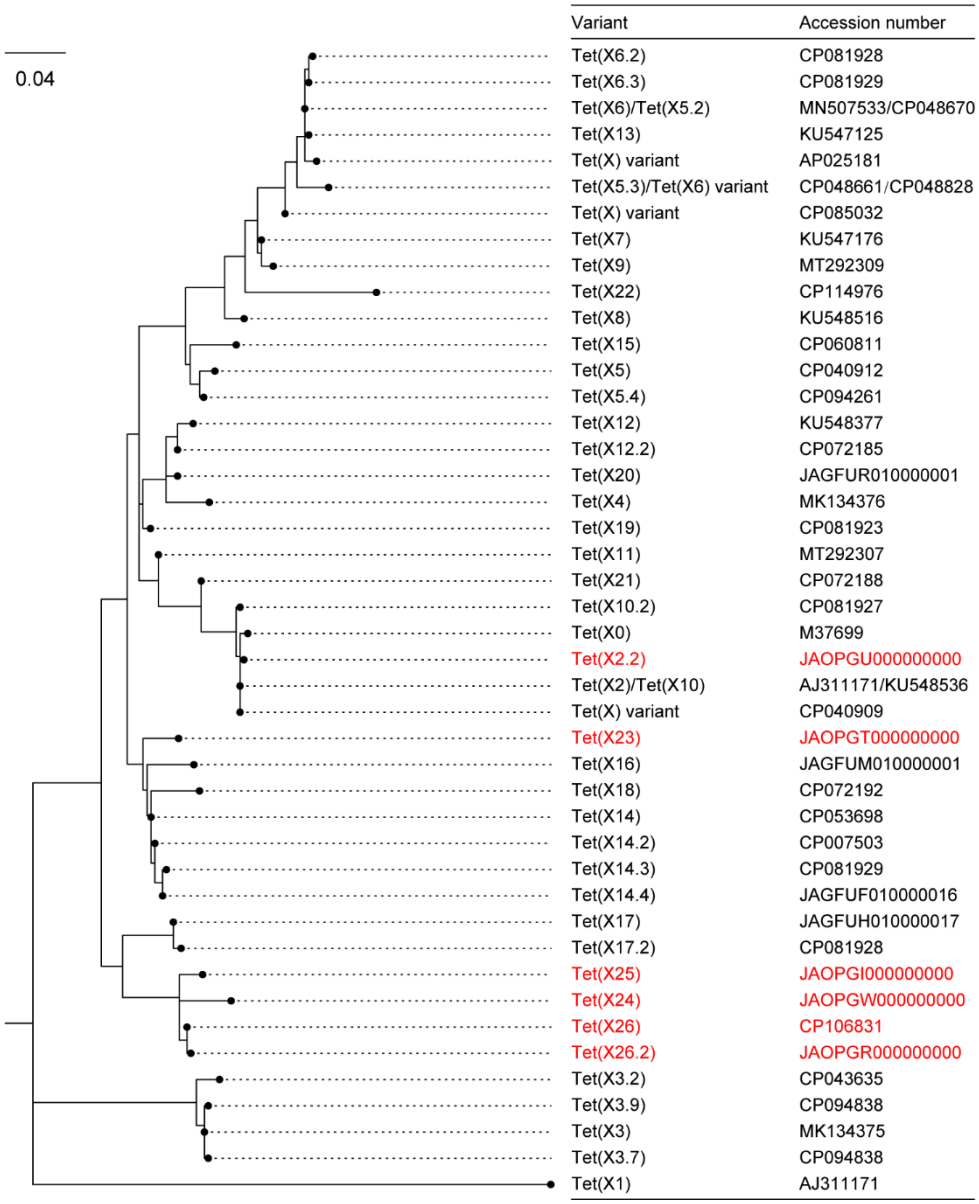

Figure S3. Maximum-likelihood phylogenetic tree of the Tet(X) variants. GenBank accession numbers are provided, of which the novel Tet(X) variants (n = 6) are marked in red. The proteins encoded by previously reported *tet*(X3.3) (CP094246), *tet*(X3.4) (CP094246), *tet*(X3.5) (CP094246), *tet*(X3.6) (CP094246), and *tet*(X3.8) (CP094838) are frameshifted, and therefore aren't involved in the phylogenetic analysis. Bar, 0.04 amino acid substitutions per site.

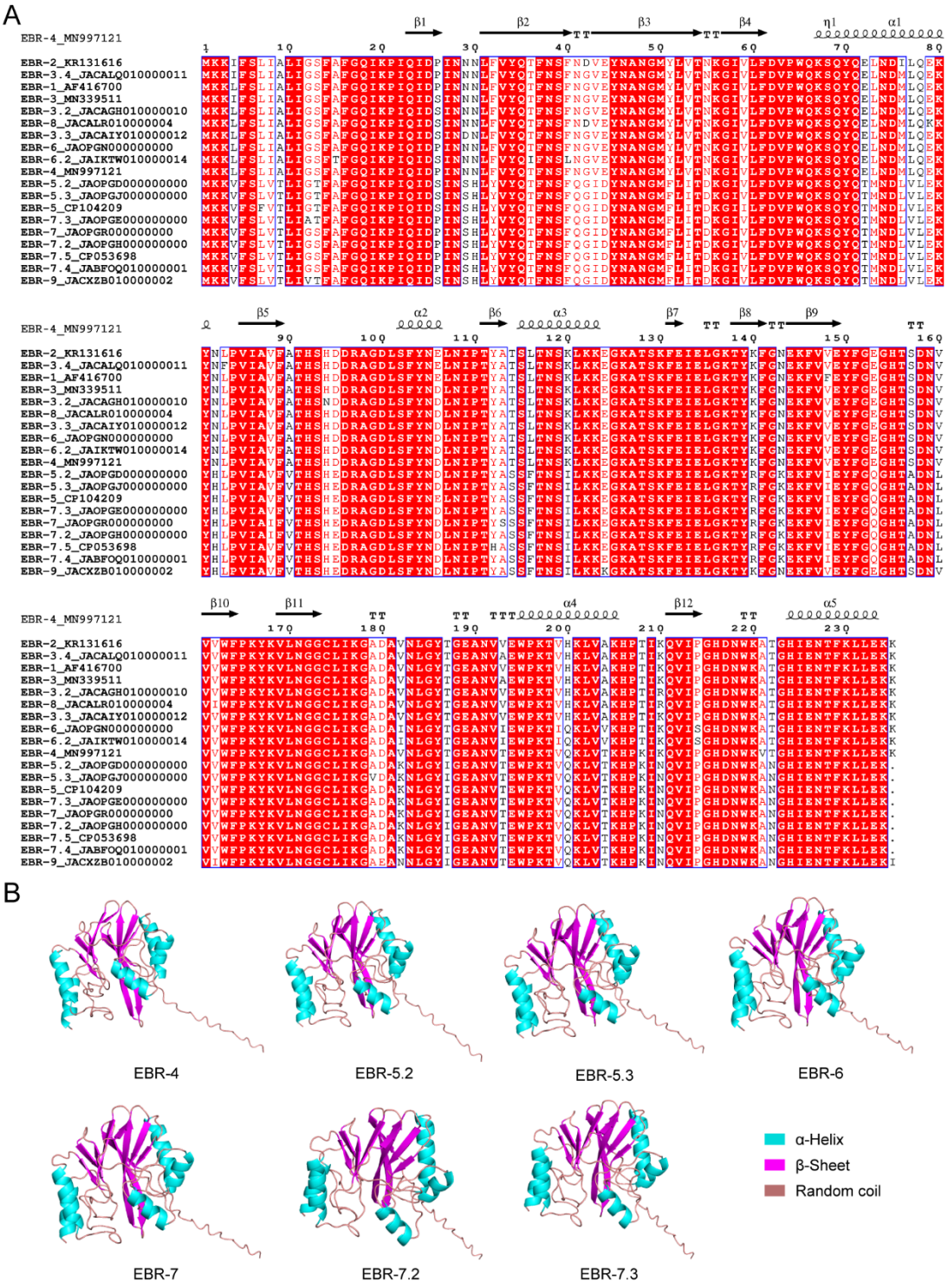

Figure S4. Structural characteristics of the EBR proteins. (A) Result of multiple **s**equence alignment by ESPript version 3.0. GenBank accession numbers of the EBR proteins are provided. (B) Prediction of three-dimensional structures by AlphaFold 3. The newly identified EBR variants are colored by their secondary structures and EBR-4 (MN997121) is used as the reference protein.

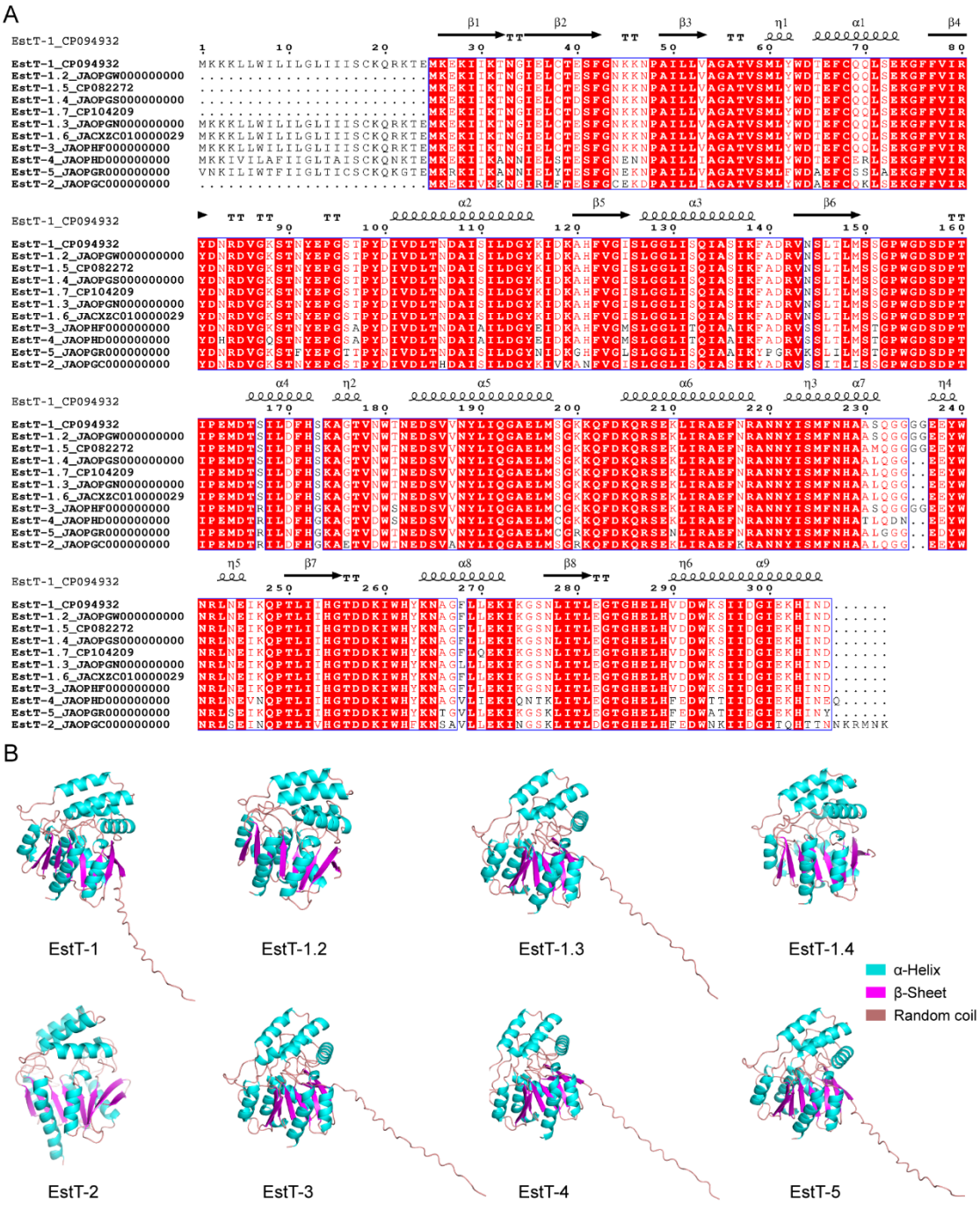

Figure S5. Structural characteristics of the EstT proteins. (A) Multiple sequence alignment of the EstT proteins by ESPript version 3.0. GenBank accession numbers of them are provided. (B) Prediction of three-dimensional structures by AlphaFold 3. The newly identified EstT variants are colored by their secondary structures and EstT-1 (CP094932) is used as the reference protein.

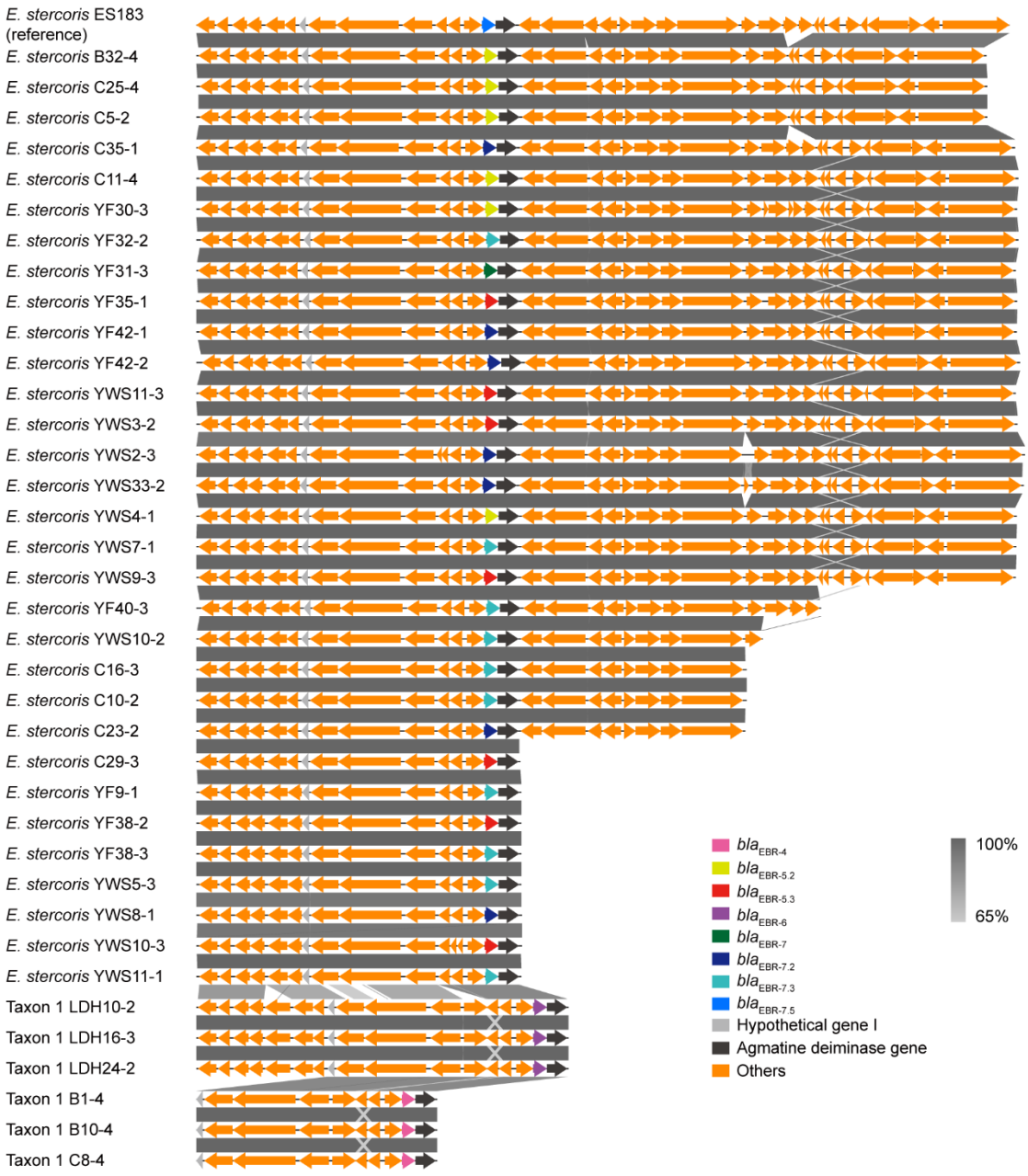

Figure S6. Conserved genetic backgrounds of the *bla*EBR genes. The *bla*EBR-7.5-positive *E. stercoris* ES183 (GCA_014621655.1) is used as the reference strain. Regions of > 65% nucleotide identity are marked by shading.

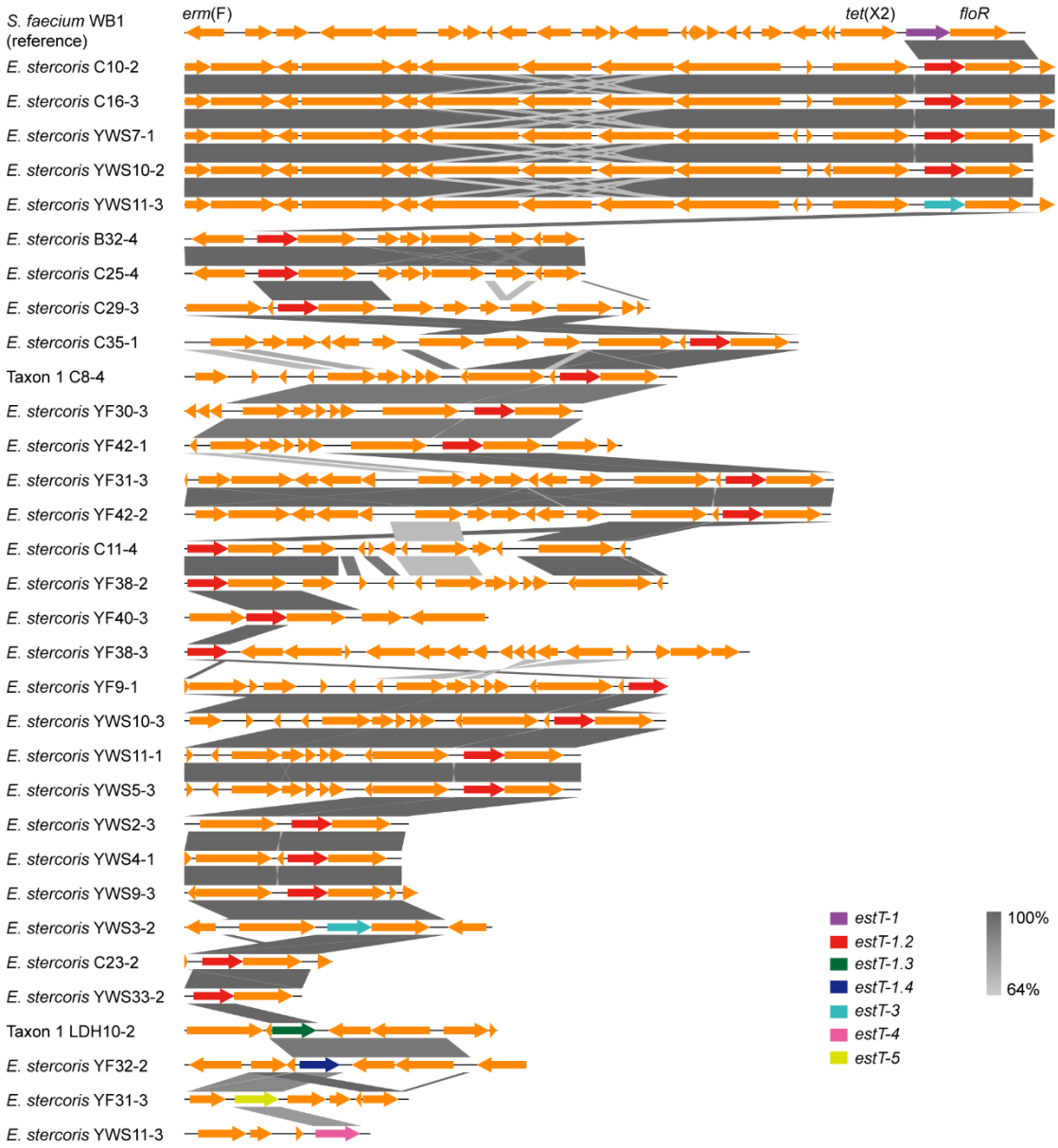

Figure S7. Diverse genetic backgrounds of *estT* genes. The *estT-1*-positive *S. faecium* WB1 (GCA_025340125.1) is used as the reference strain. Regions of > 64% nucleotide identity are marked by shading.

Table S1. Distribution of *tet*(X)- positive *Empedobacter* sp. isolates of different sources.

Province	Detection rate (percentage of positive strain/sample)				
	Chicken feces	**Chicken carcass**	**Sheep feces**	**Soil**	**Sewage**
Shandong	11.3% (22/195)	0.0% (0/60)	0.0% (0/0)	5.6% (1/18)	61.1% (11/18)
Jiangsu	2.5% (3/122)	0.0% (0/0)	0.0% (0/0)	0.0% (0/30)	0.0% (0/18)
Ningxia	0.0% (0/0)	0.0% (0/0)	0.0% (0/95)	0.0% (0/0)	0.0% (0/0)
In total	7.9% (25/317)	0.0% (0/60)	0.0% (0/95)	2.1% (1/48)	30.6% (11/36)

Table S2. Primers used in this study.

Primer	Nucleotide sequence (5′ to 3′) †	Size (bp)
tet(X)-uniF	TTCAGGTCAGGAAGCAATGAAAA	780 [1]
tet(X)-uniR	TTTACGCCTTGTCCTGCAAAAGG	
16S rDNA-F	AGAGTTTGATCCTGGCTCAG	1450 [2]
16S rDNA-R	GGTTACCTTGTTACGACTT	
tet(X2.2)-F	ttgggctagcaggaggaattcATGACAATGCGAATAGATACAGACAA	1167 ††
tet(X2.2)-R	cttgcatgcctgcaggtcgacTTATACATTTAACAATTGCTGAAACGTAA	
tet(X23)-F	ttgggctagcaggaggaattcATGAATTTACTTAGTGATAAGAACGTTGC	1137
tet(X23)-R	cttgcatgcctgcaggtcgacTTATACATTTAACAATTGCTGAAACGTAA	
tet(X24)-F	ttgggctagcaggaggaattcATGACAATGCGAATAGATACAGACAA	1167
tet(X24)-R	cttgcatgcctgcaggtcgacTTATAGATTCATTAGTTTTTGGAACGAA	
bla$_{EBR-4}$-F	ttgggctagcaggaggaattcATGAAGAAATTATTTTCACTTATAGCATTG	708 †††
bla$_{EBR-4}$-R	cttgcatgcctgcaggtcgacTTATTTCTTTTCTAAAAGTTTAAACGTATTTT	
bla$_{EBR-5.2}$-F	ttgggctagcaggaggaattcATGAAAAAAGTATTTTCTCTTGTCACATT	705 ††††
bla$_{EBR-5.2}$-R	cttgcatgcctgcaggtcgacTTATTTTTCTAAAAGTTTAAACGTATTTTCTAT	
estT-1.2-F	ttgggctagcaggaggaattcATGAAAGAGAAAATAATTAAAACAAACGG	849
estT-1.2-R	cttgcatgcctgcaggtcgacTCAGTCATTTATGTGTTTTTCTATTCCA	
estT-1.3-F	tacccgttttttttgggctagcATGAAAAAAAAACTACTTTGGATATTAATTT	915 †††††
estT-1.3-R	cttgcatgcctgcaggtcgacTCAGTCATTTATGTGTTTTTCTATTCCA	
estT-1.4-F	tacccgttttttttgggctagcATGAAAGAGAAAATAATTAAAACAAACGG	843
estT-1.4-R	cttgcatgcctgcaggtcgacTCAGTCATTTATGTGTTTTTCTATTCCA	
estT-2-F	tacccgttttttttgggctagcATGAAAGAGAAAATAGTTAAAAAAAACGG	861
estT-2-R	cttgcatgcctgcaggtcgacCTATTTGTTCATTCTTTTGTTATTTGTTG	
estT-4-F	tacccgttttttttgggctagcATGAAGAAAATAGTAATTTTAGCATTTATAATT	918
estT-4-R	cttgcatgcctgcaggtcgacTTATTGCTCATTTATATGTTTTTCAATTC	
estT-5-F	tacccgttttttttgggctagcGTGAATAAAATACTAATTTGGACTTTTATAATT	915
estT-5-R	cttgcatgcctgcaggtcgacTTAGTAATTTATGTGCTTTTCAATTCCTT	
pBAD24-F	CGGCGTCACACTTTGCTATG	Cloning confirmation [3]
pBAD24-R	ACGGCGTTTCACTTCTGAGT	

† The homologous sequences are underlined.
†† The primers are used for cloning of *tet*(X2.2), *tet*(X25), *tet*(X26), and *tet*(X26.2) genes.
††† The primers are used for cloning of *bla*$_{EBR-4}$ and *bla*$_{EBR-6}$ genes.
†††† The primers are used for cloning of *bla*$_{EBR-5.2}$, *bla*$_{EBR-5.3}$, *bla*$_{EBR-7}$, *bla*$_{EBR-7.2}$, and *bla*$_{EBR-7.3}$ genes.
††††† The primers are used for cloning of *estT-1.3* and *estT-3* genes.

Table S3. Summary of the *tet*(X) variants (n = 52, accessed on 16 Apr 2024).

Subtype	Accession number	Bacterial species	Size (bp)	Reference
tet(X0)	M37699	*Bacteroides fragilis*	1167	[4]
tet(X1)	AJ311171	*Bacteroides thetaiotaomicron*	1080	[5]
tet(X2) †	AJ311171	*Bacteroides thetaiotaomicron*	1167	[5]
tet(X2.2)	JAOPGU000000000	*Empedobacter falsenii*	1167	This study
tet(X) variant	CP040909	*Empedobacter falsenii*	1137	[6]
tet(X3)	MK134375	*Acinetobacter baumannii*	1167	[7]
tet(X3.2)	CP043635	*Empedobacter brevis*	1167	[8]
tet(X3.3) ††	CP094246	*Acinetobacter variabilis*	1166	[9]
tet(X3.4) ††	CP094246	*Acinetobacter variabilis*	1164	[9]
tet(X3.5) ††	CP094246	*Acinetobacter variabilis*	1165	[9]
tet(X3.6) ††	CP094246	*Acinetobacter variabilis*	1165	[9]
tet(X3.7)	CP094838	*Acinetobacter schindleri*	1167	[9]
tet(X3.8) ††	CP094838	*Acinetobacter schindleri*	1165	[9]
tet(X3.9)	CP094838	*Acinetobacter schindleri*	1167	[9]
tet(X4)	MK134376	*Escherichia coli*	1158	[7]
tet(X5)	CP040912	*Acinetobacter baumannii*	1167	[10]
tet(X5.2) †††	CP048670	*Acinetobacter* sp.	1137	[3]
tet(X5.3) ††††	CP048661	*Acinetobacter* sp.	1137	[3]
tet(X5.4)	CP094261	*Acinetobacter indicus*	1167	[11]
tet(X6) †††	MN507533	*Proteus* genomospecies 6	1137	[12]
tet(X6.2)	CP081928	*Riemerella anatipestifer*	1137	[13]
tet(X6.3)	CP081929	*Riemerella anatipestifer*	1137	[13]
tet(X6) variant ††††	CP048828	*Acinetobacter baumannii*	1137	[14]
tet(X) variant	AP025181	*Escherichia coli*	1137	[15]
tet(X) variant	CP085032	*Proteus terrae* subsp. cibarius	1137	[16]
tet(X7)	KU547176	Human enteric microorganism	1137	[17]
tet(X8)	KU548516	Human enteric microorganism	1137	[17]
tet(X9)	MT292309	Human enteric microorganism	1137	[17]
tet(X10) †	KU548536	Human enteric microorganism	1167	[17]
tet(X10.2)	CP081927	*Riemerella anatipestifer*	1167	[13]
tet(X11)	MT292307	Human enteric microorganism	1167	[17]
tet(X12)	KU548377	Human enteric microorganism	1167	[17]
tet(X12.2)	CP072185	*Riemerella anatipestifer*	1167	[13]
tet(X13)	KU547125	Human enteric microorganism	1137	[17]
tet(X14)	CP053698	*Empedobacter stercoris*	1167	[18]
tet(X14.2)	CP007503	*Riemerella anatipestifer*	1167	[18]
tet(X14.3)	CP081929	*Riemerella anatipestifer*	1167	[13]
tet(X14.4)	JAGFUF010000016	*Riemerella anatipestifer*	1167	[13]
tet(X15)	CP060811	*Acinetobacter variabilis*	1158	[19]
tet(X16)	JAGFUM010000001	*Riemerella anatipestifer*	1167	[13]
tet(X17)	JAGFUH010000017	*Riemerella anatipestifer*	1167	[13]
tet(X17.2)	CP081928	*Riemerella anatipestifer*	1167	[13]
tet(X18)	CP072192	*Riemerella anatipestifer*	1137	[13]
tet(X19)	CP081923	*Riemerella anatipestifer*	1167	[13]
tet(X20)	JAGFUR010000001	*Riemerella anatipestifer*	1167	[13]
tet(X21)	CP072188	*Riemerella anatipestifer*	1167	[13]
tet(X22)	CP114976	*Pseudomonas caeni*	1137	[20]
tet(X23)	JAOPGT000000000	*Empedobacter falsenii*	1137	This study
tet(X24)	JAOPGW000000000	*Empedobacter falsenii*	1167	This study

tet(X25)	JAOPGI000000000	*Empedobacter falsenii*	1167	This study
tet(X26)	CP106831	*Empedobacter falsenii*	1167	This study
tet(X26.2)	JAOPGR000000000	*Empedobacter falsenii*	1167	This study

[†] *tet*(X2) (AJ311171) and *tet*(X10) (KU548536) share an 100% nucleotide identity.

[††] The variants are frameshifted.

[††] *tet*(X5.2) (CP048670) and *tet*(X6) (MN507533) share an 100% nucleotide identity.

[††††] *tet*(X5.3) (CP048661) and the *tet*(X6) variant (CP048828) share an 100% nucleotide identity.

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